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Detection of Alterations in Testicular and Epididymal Function in Laboratory Animals

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The potential impact of an agent altering male reproductive function is greater for humans than for animals. Consequently, it is essential that sensitive criteria be used to look for effects on a multiplicity of target sites when an agent is evaluated using an animal model. No animal model has reproductive characteristics similar to those of humans, but this does not negate the validity of using animal models. Classic methodologies for reproductive toxicology are limited by the approaches used for subjective evaluation of testicular histology and use of natural mating for fertility tests. After dosing for an interval at least equal to six times the duration of one cycle of the seminiferous epithelium, sperm from ejaculated semen or the cauda epididymidis can be evaluated for normalcy of morphology or function and should be used for artificial insemination of females to critically evaluate fertility. Normal males of animal models ejaculate a great excess of sperm. A 50 or 90% reduction in the number of fertile sperm deposited during mating probably will not markedly reduce fertility. Artificial insemination of a critical number of sperm, selected to result in slightly less than maximal fertility for control animals, will maximize the probability of detecting a decrease in fertility if the same critical number of sperm is inseminated for treated animals as for control animals. Testicular function should be evaluated by objective, rather than subjective, criteria. For each male, a piece of testicular tissue should be appropriately fixed and an aliquot of parenchyma should be homogenized to allow enumeration of homogenization-resistant spermatids. Among the more sensitive criteria of testicular function are the minor diameter of essentially round seminiferous tubules, the ratio of leptotene spermatocytes to Sertoli cells, the corrected numbers of germ cells per seminiferous tubule cross section, and the number of homogenization-resistant spermatids per testis.

Introduction

The concept of using animal models to detect changes in male reproductive function induced by a drug or environmental toxin is not new. During the past six years, however, there has been an increasing awareness that conventional toxicity tests might not be as sensitive as the techniques of modern andrology and often not cost-effective (*1–6*).

A major endpoint in a conventional test of a potential toxin for effects on reproductive function is siring offspring. Typically, a male is allowed to copulate with one or more females, and birth of live offspring is taken as evidence that the test agent does not affect reproductive function. However, normal males of model species produce an ejaculate containing 10 to 1000 times as many sperm as necessary for normal fertility and litter size. A 90% reduction of the number of fertile sperm available for ejaculation, by surgical alteration of rats, did not depress fertility (*7*). If an agent administered to adult rats induced a similar decrease in the number of fertile sperm ejaculated during copulation, the alteration of reproductive function would be insufficient to cause a demonstrable decrease in fertility or litter size (*2,8*). Thus, the conventional fertility test is insensitive.

The potential impact of an agent affecting male reproductive function probably is greater for humans than for animals. When expressed on the basis of pregnancies per cycle of intentional exposure of a normal female by a normal cohabitating male, the fertility of humans is considerably lower than for common mammals. Although accurate data for humans are not available, it is estimated that only about one pregnancy per year would result from unprotected intercourse during normal cohabitation and less than one-third of all fertilized eggs result in a full-term gestation. With most laboratory or domestic mammals, a live birth results from at least 50% of the females exposed to a male during one estrous cycle. There are many reasons for this difference, and they will not be considered herein. The crucial point is that reproductive function in humans is uniquely less efficient than in common mammals. Therefore, it is prudent to assume that many perturbations of reproductive function that will not affect birth rate in a population of infrahuman mammals could cause reproductive failure in humans.

This discussion is restricted to a consideration of postpubertal animals. However, it is obvious that prenatal, infantile, or prepubertal exposure of a male to a toxic

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agent could affect subsequent reproductive performance. However, the design of most experiments precludes a critical evaluation of sequelae to exposing a male to a given agent during a short interval prior to the onset of spermatogenesis. The design of such experiments is especially difficult because the interval between birth and events triggering prepubertal development of the hypothalamic-pituitary axis, Leydig cells, or Sertoli cells, with resultant initiation of spermatogenesis, is extremely short (*9*). In rodents, triggering signals may occur before birth and certainly occur prior to weaning. The relative sensitivity to representative agents of the germinal epithelium in infants and men is not established. Consequently, even if appropriate animal data were available, there would be little basis for the necessary extrapolations from animal models to man.

Selection of an Animal Model

No animal model has reproductive characteristics similar to those of humans (Table 1). However, this does not negate the validity of using animal models to screen agents for effects on male reproductive function. The efficiency of sperm production is higher in males of all species likely to be used as an animal model than in the human (20–28 $\times$ 10^6 vs. 4 $\times$ 10^6 sperm/g testis/day), but this difference may be advantageous since it allows a greater range of response. Similarly, the uniformity of the germinal epithelium, or morphology of ejaculated sperm, results in a low coefficient of variability for many characteristics likely to be evaluated in animal models. Since the variability for such characteristics among normal males of animal models tends to be less than for men, detection of a deviation from normal would require fewer rats or rabbits than would be true if similar evaluations on humans were possible.

Among animal models likely to be used (Table 1), rats are preferable to mice or hamsters because of their convenient size, well-characterized reproductive processes, and general use in toxologic studies. The reproductive organs of rats are large enough that sufficient tissue is available to examine biochemically or morphologically even after a 50 to 75% reduction in weight. However, mice have been used advantageously to study the effect of chemotherapeutic drugs on testicular function (*10*). A disadvantage of rats and similar rodents is that semen or spermatozoa from a given male cannot be evaluated in a longitudinal study.

Rabbits are an ideal second species because they are the smallest common species for which semen can be collected quantitatively and conveniently in longitudinal studies (*11–13*). Necessary background information on rabbits is available (*2*). With both rats (or mice) and rabbits, fertility testing is convenient, and artificial insemination is practical.

The dog is less desirable as an animal model. The size of a dog facilitates hormonal or biochemical analysis of blood, and longitudinal collection of semen is possible. However, fertility testing is virtually impossible. The

Table 1. Potential models for studying alterations of male reproduction.[a]

	Mouse	Hamster	Rat	Rabbit	Dog (beagle)	Monkey (rhesus)	Man
Duration of cycle of seminiferous epithelium, days	8.9	8.7	12.9	10.7	13.6	9.5	16.0
Lifespan, days							
B-spermatogonia	1.5	1.6	2.0	1.3	4.0	2.9	6.3
Leptotene spermatocytes	2.0	0.8	1.7	2.2	3.8	2.1	3.8
Pachytene spermatocytes	8.0	8.1	11.9	10.7	12.4	9.5	12.6
Golgi spermatids	1.7	2.3	2.9	2.1	6.9	1.8	7.9
Cap spermatids	3.6	3.5	5.0	5.2	3.0	3.7	1.6
Fraction of lifespan as							
B-spermatogonia	0.11	0.12	0.10	0.08	0.19	0.19	0.25
Primary spermatocyte	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Round spermatid	0.41	0.46	0.40	0.43	0.48	0.35	0.38
Testes wt, g	0.2	3.0	3.7	6.4	12.0	49	34
Daily sperm production							
Per gram testis, 10^6/g	28	24	24	25	20	23	4.4
Per male, 10^6	5	74	86	160	290	1100	125
Sperm reserves in cauda (paired sides at sexual rest), 10^6	49	1020	440	1600	2100	5700	420
Epididymal transit (at sexual rest), days		14.8	8.1	12.7	11.3	10.5	5.5
Evaluation possible of							
Testis size *in situ*	No	Yes	Yes	Yes	Yes	?	Yes
Enumerate testis spermatids	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Evaluate testis histology	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Quantitatively collect semen	No	No	No	Yes	Yes	?	?
Fertility tests feasible							
Natural mating	Yes	Yes	Yes	Yes	No	No	No
Artificial insemination	Yes[b]	Yes[b]	Yes[b]	Yes	No	No	No

[a] Data compiled from the literature.
[b] Artificial insemination with sperm from the cauda epididymidis is practical.

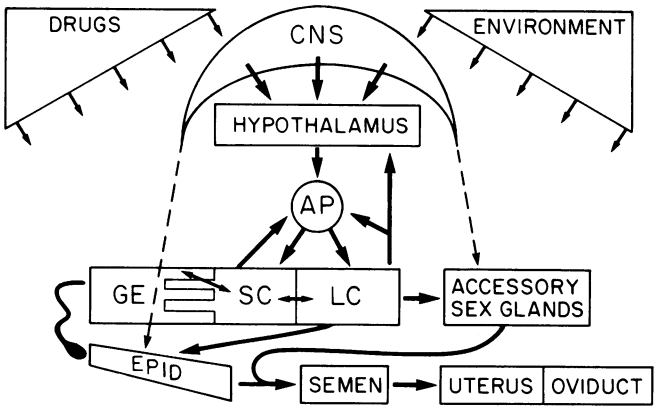

FIGURE 1. Interdependence of components of the male reproductive system.

3- to 9-month postpartum anestrous of the bitch and our inability to reliably induce estrus or ovulation make fertility testing in dogs very expensive. Widespread use of sub-human primates for routine testing is unlikely. Rarely, if ever, would the nature of an agent preclude evaluation in a species such as mice, rats, rabbits, or dogs and necessitate use of subhuman primates. In considering animal models, it is conventional to restrict one's thinking to mammals. However, use of an avian model, such as the chicken (*14*) might be advantageous. Toxic effects on sperm of at least one common environmental agent (caffeine) have been detected by lowered fertility of hens inseminated with normal-appearing sperm (*14*).

Potential Sites of Action of an Agent in the Male

Normal male reproductive function requires neural communication between the central nervous system and the hypothalamus or reproductive organs. Hormonal and neurochemical signals relay information among the hypothalamus, anterior pituitary gland, Leydig cells, Sertoli cells, and germinal epithelium (Fig. 1). Thus, the function of each of these components of the male reproductive system, as well as the epididymis, accessory sex glands, and ejaculatory mechanism is controlled by signals from one or more distant sites. In theory, fertility and fecundity might be suppressed by altered function at any of the sites listed in Table 2. However, in many cases, a relatively minor but physiologically important alteration in one or more of the listed mechanisms of action might not result in reduced fertility or sterility. Nevertheless, such changes in an animal model should be sought and considered in risk assessment.

Presumably, the objective of a toxicity test is to establish that an agent probably has no adverse effect on reproductive function. Alternatively, the objective might be to determine the dose response of altered reproductive function following administration of an agent. In either case, it is essential to use the most sensitive criteria for evaluating reproductive function. Tests listed in Table 2 fall into several categories: analyses of hormone receptors, analyses of hormone concentrations in blood or tissue, quantitative morphologic evaluations of reproductive tissues, measurement of sperm production, examination of seminal characteristics, and determination of fertilization rate or litter size. For the next several years, it is likely that evaluation of testicular or epididymal function and of seminal characteristics will continue to be the primary approaches to detect effects of toxins on male reproductive function. Limitations of classic methodologies are not in the tissues and fluids examined, but rather with the approaches of subjective evaluation of testicular histology or a fertility test utilizing natural mating. However, gradual introduction of other approaches (such as in Table 2) seems inevitable and is desirable.

Use of cultured cells, either primary isolations or stabilized lines representing important components of the male reproductive system, to evaluate potential reproductive toxins certainly will increase. Similarly, certain effects of an agent can be evaluated by directly exposing sperm to a low concentration of the agent. However, these approaches must be used cautiously, since they circumvent many aspects of the normal physiologic processes involved in the production and delivery of fertile sperm to the site of fertilization within the female reproductive system. A greater incidence of false negative responses must be expected if use of whole animal models is excluded.

An agent acting directly on the brain, hypothalamus, or anterior pituitary gland will indirectly affect the testes and possibly will affect sexual behavior. A neural effect also might alter transport of sperm through the epididymis, emission, or ejaculation. An agent acting on the testes would indirectly affect the epididymides, accessory sex glands, and seminal characteristics, as well as the hypothalamus and anterior pituitary gland. An agent acting directly on the epididymides or passed directly into epididymal fluid would affect the spermatozoa, but probably would not affect other components of the reproductive system (Fig. 1). Similarly, an agent acting directly on the accessory sex glands or transferred into seminal plasma could alter spermatozoal function, but probably would not alter other aspects of male reproductive function.

An agent—or metabolite of the agent—administered to an animal would be distributed to most components of the male reproductive system via the blood and tissue fluid. However, the seminiferous tubules of a normal male are characterized by the presence of a "blood–testis barrier." Tight junctions among adjacent Sertoli cells divide the germinal epithelium into two functional compartments: the basal compartment and the adluminal compartment. The basal compartment contains all spermatogonia as well as preleptotene and early leptotene spermatocytes and, thus, is the site of all germ cells synthesizing DNA or undergoing mitosis. Most agents probably would have unrestricted access to the

Table 2. Potential sites and mechanisms of action of toxic agents in the adult male and approaches for detecting altered reproductive function in males.[a]

Site of action	Potentially altered mechanisms	Evaluative tests
Hypothalamus	Neurotransmission	None at present (?)
	Synthesis and secretion of GnRH	Hormone assay
	Receptors for LH, FSH, and steroids	Receptor analyses
Anterior pituitary gland	Synthesis and secretion of LH, FSH, and PRL	Hormone or mRNA assays and GnRH challenge
	Receptors for GnRH, LH, FSH and steroids	Receptor analyses
Testis	Receptors for LH and PRL on Leydig cells	Receptor analyses
	Testosterone synthesis and secretion	*In vitro* production and hormone assay
	Vascular bed or blood flow	Morphology and ??
	Blood-testis barrier	Morphology and micropuncture
	Receptors for FSH on Sertoli cells	Receptor analyses
	Receptors for steroids	Receptor analyses
	Secretion of inhibin or ABP	*In vitro* tests and assay
	Sertoli cell function	*In vitro* tests
	Death of reserve spermatogonia	Germ cell counts
	Spermatogonial mitosis	Germ cell counts and % tubules without germ cells
	Spermatocyte meiosis	Spermatid counts and % tubules with luminal sperm
	Spermatid differentiation	Sperm morphology
	Daily sperm production	Count spermatids and seminal evaluations
Efferent ducts	Vascular bed	Morphology
	Resorption	???
Epididymis	Resorption	Sperm maturation
	Concentration of blood constituents	Sperm maturation and biochemical analyses
	Secretion and interconversions	Biochemical analyses
	Enzyme activity	Biochemical analyses
	Transfer of agent to luminal fluid	Assay for agent
	Smooth muscle contractility	Response to drugs *in vivo* or *in vitro*
	Sperm transport	Sperm in ejaculate
Ductus deferens	Smooth muscle contractility	Response to drugs *in vivo* or *in vitro*
	Sperm transport	Sperm in ejaculate
Accessory sex gland	Secretion of agent	Assay for agent
	Secretion of spermicidal products	Evaluate sperm motility
Semen	Presence of agent	Assay for agent
	Spermicidal components	Evaluate sperm motility

[a]Abbreviations used: GnRH, gonadotrophin releasing hormone; LH, luteinizing hormone; FSH, follicle stimulating hormone; and PRL, prolactin. Modified from Amann (*2*).

basal compartment. The adluminal compartment contains primary spermatocytes more mature than leptotene, all secondary spermatocytes, and all spermatids. Thus, the major portion of meiosis, including both meiotic divisions, and the morphologic transformations of spermatids, occur within the adluminal compartment where the environment around germ cells of a given type is directly controlled by the surrounding Sertoli cells. Except for agents that cause destruction of the blood-testis barrier, access to the adluminal compartment is controlled by the Sertoli cells. Micropuncture studies (*16*) have revealed that many agents are rapidly transferred by Sertoli cells into the fluid within the seminiferous tubule lumen, whereas other agents are selectively excluded. In the epididymis, junctional complexes near the apical face of the principal cells of the epithelium lining the duct similarly form a "blood-epididymis barrier" which also restricts entrance of many molecules into the lumen of the ductus epididymides (*16*). Nevertheless, it is evident that many agents pass rapidly from the blood into the epididymal plasma and, thereby, can adversely affect spermatozoal function. Similarly, the accessory sex glands can serve as a rapid transfer point for movement of an agent from blood into seminal plasma where it might influence spermatozoal function.

Spermatogenesis

An understanding of spermatogenesis is essential for evaluation of testicular histology and function. Although published a decade ago, several excellent reviews (*17–20*) of spermatogenesis remain timely. Within a seminiferous tubule, there are two populations of spermatogonia. One, termed reserve spermatogonia, is extremely resistant to radiation or toxic effects and often survives exposure to an agent that kills the proliferating germ cells; restoration of spermatogenesis may occur after clearance of the compound inducing aspermatogenesis. Resistance of reserve spermatogonia to mitotic or DNA synthesis inhibitors is a result of their very long cell cycle time. The other population of spermatogonia serves as the source of the proliferating pool of germ cells. Cells termed stem A_1-spermatogonia in one area of a seminiferous tubule synchronously become committed to produce increasingly differentiated spermatogonia. Consequently, cohorts of cells, resulting from these A_1-spermatogonia, differentiate in unison

and there is a synchronous population of developing germ cells at that area within the seminiferous tubule.

At a given point within a seminiferous tubule, groups of A_1-spermatogonia become differentiated, and thereby initiate development of cohorts of cells which proceed towards formation of spermatozoa, at a fixed interval. This interval is equivalent to the duration of one cycle of the seminiferous epithelium and is a characteristic of a given species. For common animal models, the duration of one cycle of the seminiferous epithelium ranges from 8.9 days to 13.6 days (Table 1), although it is 16.0 days for man.

It takes considerably longer than the duration of one cycle of the seminiferous epithelium for a cohort of germ cells to progress from a differentiated A_1-spermatogonium, through a series of increasingly differentiated spermatogonia, primary spermatocytes, secondary spermatocytes and spermatids, to spermatozoa. Consequently, at a given point within a seminiferous tubule, different cohorts of germ cells, representing successively less mature cell types develop simultaneously but separated in time by the duration of one cycle of the seminiferous epithelium. Because the duration of spermatogenesis is approximately 4.5 times the duration of one cycle of the seminiferous epithelium, in a given cross section through a normal seminiferous tubule there are four or five generations (cohorts) of developing germ cells. In a normal testis, the type or degree of differentiation of germ cells of a given cohort, relative to the type or degree of differentiation for cells in preceding and succeeding cohorts, is remarkably uniform. Thus, it is possible to classify the appearance of the germinal epithelium into a series of cellular associations (stages) which represent characteristic groupings of germ cells.

Since the cellular associations represent a human classification of a continuous process into a series of discrete stages, it is not surprising that differing approaches have been used. Of the two conventional approaches for classifying cellular associations (*15,18*), one relies on the identification of germ cells within the several generations present at a given area within a seminiferous tubule (typically the major portion of a cross section through a seminiferous tubule has a similar appearance). The other approach is to base the classification on the morphology of the acrosome within developing spermatids.

For most common mammals, adjacent cross sections through seminiferous tubules usually look different. This is normal. Recognition of this fact is imperative for either qualitative or quantitative histologic evaluation of testicular tissue. Depending on the species and classification scheme, 6 to 14 different cellular associations have been discerned (*15,19,20*). Each cellular association contains four or five generations (or types) of germ cells organized in a specific, layered fashion (Fig. 2). The complete series of cellular associations is termed the cycle of the seminiferous epithelium. Since the cellular associations making up the cycle of the seminiferous epithelium represent the developmental changes

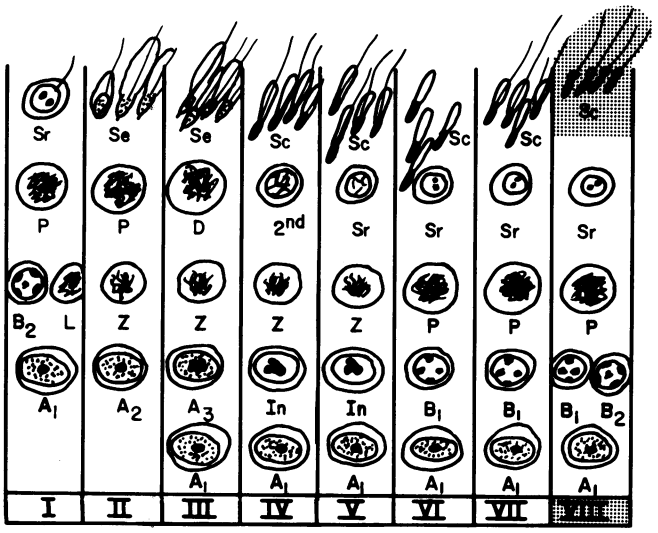

FIGURE 2. Cellular associations (I to VIII) of the cycle of the seminiferous epithelium of an animal model. The germ cells in each of the four or five generations (cohorts) present in each cellular association are depicted from the basement membrane (bottom) to the lumen (top). The complete sequence of cellular associations (Stages) occurs at a given point in a seminiferous tubule over an interval termed the duration of the cycle of the seminiferous epithelium (Table 1). Secondary spermatocytes are found only in cellular association IV and mature spermatids are found lining the lumen of the of the seminiferous tubule only in cellular association (Stage) VIII. The relative durations of the cellular associations, including that of cellular association VIII, differs among species (Table 3).

occurring concurrently to several cohorts of germ cells at a fixed point in a seminiferous tubule, at a given point within a seminiferous tubule there is a sequential progression through the cycle of the seminiferous epithelium.

The relative duration of each cellular association (or stage) is a species characteristic. Only one cell association contains secondary spermatocytes, and these cells have a short life before they complete meiosis. This cell association is uniformly short for all species. Depending on the classification scheme, one or two cellular associations are characterized by mature spermatids lining the tubule lumen (cell association VIII in Fig. 2). Cross sections through such tubules are rare in rabbits (8%) but common in rats (38%) (Table 3).

Evaluation of Testicular Function

With the preceding exposure to the kinetics of spermatogenesis, and knowledge that the duration of spermatogenesis ranges from 40 days in mice to 62 days in dogs or 72 days in men (4.5 times the duration of one cycle of the seminiferous epithelium), it is possible to consider approaches to evaluating testicular function. A strategy of continuous dosing will maximize the probability of detecting an alteration of spermatogenesis, or any other aspect of male reproduction function, if indeed

Table 3. Frequency of occurrence (%) of cross sections through seminiferous tubules having elongated spermatids with condensed nuclei (cellular association V to VIII) or mature spermatids lining the tubule lumen (cellular association VIII).[a]

Species	Tubule cross sections, %	
	In V to VIII	In VIII
Rabbit	38	8
Dog	52	14
Hamster	67	19
Rhesus monkey	42	26
Mouse	69	36
Rat	70	38

[a] Compiled from the literature. See Figure 2 for the germ cells present in cellular associations V to VIII or in cellular association VIII.

one occurs. Males should be dosed for an interval at least equal to six times the duration of one cycle of the seminiferous epithelium. At that time, sperm from ejaculated semen or recovered from the cauda epididymidis can be used for artificial insemination of females to critically evaluate fertility. Aliquots of the same samples can be used to evaluate spermatozoal morphology in stained smears or by phase-contrast microscopy of wet preparations. Testicular function can be evaluated with a high probability of detecting an alteration if one exists. If an agent is found to affect spermatogenesis, studies using brief administration of the agent will be necessary to establish the site of action of the agent (*21*).

The cells most sensitive to chemotherapeutic drugs or radiation are those undergoing mitosis or synthesizing DNA, namely, the proliferating spermatogonia or preleptotene primary spermatocytes (*22–24*). However, there are exceptions to this generalization: other types of drugs or environmental toxins, including heat and inhibitors of RNA synthesis, affect the more differentiated germ cells (*15,21,25*). It is uncertain if agents cytotoxic to spermatocytes or spermatids, or inducing aberrant spermatozoal morphology or function, act directly on these germ cells or indirectly by altering Sertoli cell function which, in turn, provides an abnormal microenvironment incompatible with spermatogenesis or prevents necessary Sertoli cell functions associated with spermiogenesis. Given the characteristic alterations seen in the seminiferous tubules after exposure to a variety of toxic agents (*23*) and the nature of the blood-testis barrier and spermatogenesis, it is prudent to assume that many agents deleterious to spermatogenesis act directly on Sertoli cells and that death or malformation of germ cells often are secondary sequela to abnormal Sertoli cell function.

If there is a transitory exposure of a male to an agent for a few hours or a few days, such exposure may result in death of specific types of germ cells. Consequently, germ cells more mature than those affected by the agent will proceed through spermatogenesis and, in many cases, cohorts of germ cells less mature than those affected by the agent will develop from newly committed A_1-spermatogonia. Consequently, if one examines the testis several days or several weeks following such an insult, a casual examination may overlook the presence of a missing generation (or cohort) of germ cells in seminiferous tubules of one or more specific cellular associations. With short exposure to a mild toxin, destruction of the major portion of the germinal epithelium may not occur. However, with continuous exposure to the same toxin, all germ cells of the affected type are altered or killed as they are formed on successive days. When dosing has continued for an interval sufficiently long to allow completion of spermatogenesis by all germ cells more mature than those affected by the agent, there will be an obvious absence of, for example, late pachytene primary spermatocytes, diplotene primary spermatocytes, secondary spermatocytes and spermatids. Such a defect is readily apparent. Continued exposure to an agent blocking spermatogonial mitosis will result in a germinal epithelium containing only Sertoli cells, a few A_1-spermatogonia, and reserve spermatogonia. Depending on the agent, it is possible that the reserve spermatogonia may survive, to a varying degree, or may be completely destroyed (*10*).

Most agents do not alter the temporal pattern of spermatogenesis. Cell death or malformation occurs, but the interval between commitment of an A_1-spermatogonium and development of the cohort of germ cells to a specific point of development is not altered. However, certain drugs cause a delay in spermatid development (*21,26*). Thus, it should not be assumed that the timing of spermatogenesis always will be normal.

If the Sertoli cells have not been irreversibly damaged by the agent and if A_1-spermatogonia or reserve spermatogonia remain, there is a high probability that partial, if not complete, recovery of the germinal epithelium will follow cessation of exposure to the agent. However, deleterious effects of an agent may continue for some time following cessation of exposure. Furthermore, the nature of spermatogenesis dictates that an interval greater than the duration of spermatogenesis in that species will be required for restoration of spermatogenesis. Typically, an interval of at least 3 to 6 months should be allowed for maximum recovery of spermatogenesis, and several years may be required in humans (*10,24,27*).

There are two basic approaches for evaluating the effects of an agent on testicular function. These are evaluation of the testicular parenchyma or the characteristics of ejaculated semen. With rabbits or dogs, semen can be collected in a longitudinal study and the total number of sperm in each ejaculate and the morphology of sperm in selected ejaculates should be determined (*11,13,28,29*). The number of sperm in an ejaculate is influenced by many factors including age, testicular size, ejaculation frequency or interval since the preceding ejaculation, and the degree of sexual arousal (*2,8,30*).

In a longitudinal study, a short and uniform interval between successive ejaculates is essential for two reasons. The first reason for the interval is so that data on the mean number of sperm in an ejaculate, or ejaculated weekly, will accurately reflect the daily sperm produc-

Table 4. Effect of ejaculation regimen on weekly sperm output and components of variance.[a]

Ejaculation regimen	Sperm/wk, 10^6	Components of variance, %	
		Within males	Among males
1 ejac/wk	273	65	35
4 ejac 1 day/wk[b]	543	56	44
1 ejac daily	560	35	70

[a]Modified from Desjardins et al. (*28*).
[b]Four ejaculates collected during 30–50 min on the same day each week.

tion (*8,30*). The number of sperm in an isolated ejaculate or even in several ejaculates collected at infrequent or irregular intervals provides little information other than that the male had produced sperm during the past month. Second, collection of semen with a short and uniform interval between ejaculations is necessary to reduce variation among ejaculates within males in the total number of sperm ejaculated (*28,29*).

For rabbits from which one ejaculate was collected every 7 days, 65% of the variation was associated with weeks, which reflects variation among ejaculates from a given male (Table 4). Only 35% of the total variance was associated with differences among males. When the same rabbits were ejaculated once daily, the variance associated with weeks within males was small and that associated with males (70%) was the major component. Under this latter condition, if the males were assigned to treatment groups (ideally on the basis of pre-experimental daily sperm output), there is a reasonable probability of detecting a treatment effect. With only one ejaculate weekly, the probability of detecting a treatment effect is very low because most of the variance would be associated with ejaculates within males rather than among males. With infrequent ejaculations, a 50% reduction in sperm production, such as induced by hemicastration or unilateral vasectomy, probably would not be detected on the basis of seminal characteristics (*8*).

In most initial screens of potentially toxic agents, it is likely that a rodent model will be used and longitudinal collection of semen would not be a viable option. The appropriate strategy would be to administer the agent for at least six times the duration of one cycle of the seminiferous epithelium (*2*) and then kill the animals for a cross-sectional analysis of reproductive function and fertility. Body and organ weights should be recorded. At least one caput-corpus epididymidis from each male should be homogenized (*31*) to allow enumeration of the number of sperm therein, which provides information on the combined effects of sperm production and transit of sperm through the epididymis. Similar enumeration of the number of sperm in the cauda epididymidis is less meaningful because the number of sperm stored in the cauda epididymidis is greatly influenced by the number of copulations occurring within the previous 7 to 10 days (*8,30*).

Aliquots of testicular tissue should be fixed in an appropriate fixative (*2,5*); use of 10% buffered formaldehyde followed by embedment in paraffin is unsatisfactory. Bouin or Zenker fixatives followed by paraffin embedment or aldehyde fixation followed by plastic embedment should be used (*5*). Another sample of testicular parenchyma should be weighed and homogenized (*30–32*) to allow enumeration of elongated spermatids resistant to homogenization. The nucleoprotein of spermatids becomes highly condensed and extremely resistant to mechanical or biochemical breakdown during the latter phases of spermiogenesis. Thus, homogenization destroys all cells and nuclei within the testis except for the homogenization-resistant spermatid nuclei (or spermatozoa when epididymal tissue is homogenized). Following appropriate homogenization and dilution, the number of homogenization-resistant spermatids in an aliquot of testicular tissue can be counted using a conventional cytometer chamber and a phase-contrast microscope. This simple technique has been shown to be useful and sensitive in studies evaluating damage to the germinal epithelium of mice (*10*) and rats (*33*).

Numerous approaches for objective evaluation of testicular histology were evaluated in recent studies with rats (*33*) and rabbits (*34*) administered dibromochloropropane (DBCP) as a test agent. Among the more sensitive criteria were the minor diameter of essentially round seminiferous tubules, the ratio of leptotene spermatocytes to Sertoli cells, and the corrected numbers of spermatogonia, preleptotene spermatocytes, pachytene spermatocytes, or round spermatids per seminiferous tubule cross section. Although the same doses of DBCP were used for the rats and rabbits, and quantitative evaluations of testicular histology for both species were made by the same individual, the effects of a given dose of DBCP in the rat were less dramatic than those in the rabbit (Table 5). For the rats, testicular weight was not reduced by administration of DBCP, but there were significant differences in diameter of the seminiferous tubules, the ratio of leptotene spermatocytes to Sertoli cells, and daily sperm production (calculated from the number of homogenization-resistant spermatids). These same measures, as well as the numbers of germ cells in Stage I seminiferous tubules, also revealed a treatment effect in the rabbits. For both the rats and rabbits, fertility was not suppressed even at the highest dose of DBCP; for neither species was artificial insemination using a critical number of sperm per insemination used to evaluate fertility.

Data on quantitative histologic evaluations of the rat and rabbit testes studied by Amann and Berndtson (*33*) and Foote et al. (*34*), are being used to determine the minimum numbers of observations necessary to get precise data. Based on preliminary analyses (Berndtson et al., unpublished data), it appears that measurement of seminiferous tubule diameter can be based on only 15 tubules per testis, and counts of germ cells in tubule cross sections of a specific cellular association can be based on only three or four tubules. However, it is crucial that the few cross sections evaluated be at distant sites in the testis so that they do not represent multiple sections through the same tubule.

Table 5. Effects of DBCP on the testes of rats and rabbits.[a]

Characteristics	Rats, DBCP, mg/kg			Rabbits, DBCP, mg/kg		
	0	3.8	15.0	0	3.8	15.0
Paired testes wt, g	4.0	3.9	3.6	3.4[d]	3.0[d]	1.5[e]
S. tubule diameter, μm	212[d]	209[de]	199[e]	146[d]	133[d]	103[e]
S. tubules with elongated spermatids, %	88	89	90	61	63	44
Leptetene cytes: Sertoli cells	2.0[d]	1.9[de]	1.7[e]	2.8[d]	1.9[e]	0.8[f]
Germ cells/s. tubule cross section[c]						
Spermatogonia	2.5	2.1	1.8	2.3[d]	1.6[e]	1.0[f]
Preleptetene spermatocytes	53	55	48	42[d]	29[e]	14[f]
Pachytene spermatocytes	52	53	49	39[d]	30[e]	11[f]
Round spermatids	215	218	208	141[d]	85[d]	37[e]
Daily sperm production (10^6/testis)	42[d]	42[d]	38[e]			

[a]From Ammann and Berndtson (*33*) and Foote et al. (*34*). Within a species, means on the same row with a different superscript (d,e,f) differ ($p < 0.05$), but the significance is based on data for all seven dose groups in the experiments, rather than only the three shown.
[b]The percentage of seminiferous tubules containing step 12–16 spermatids for rats or stage IV to VIII spermatids for rabbits.
[c]Corrected numbers of germ cells per stage VIII seminiferous tubule cross section for rats or stage I seminiferous tubule cross section for rabbits.

Table 6. Percentage of rats in a dose group with values less than the designated value characteristic of normal rats.[a]

Daily dose DBCP, mg/kg	Daily sperm production < 39 × 10^6/testis	Leptotene spermatocytes: Sertoli cell ratio ≤ 1.6
0.0	13	0
0.9	21	8
1.9	19	7
3.8	35	13
7.5	24	21
15.0	60	40

[a]From Amann and Berndtson (*33*).

Inspection of data for control rats in the DBCP experiment revealed that most had a daily sperm production ≥ 39 × 10^6/testis and a ratio of leptotene spermatocytes to Sertoli cells of ≥ 1.6 (*33*). Consequently, the percentages of rats in each treatment group having lower values were calculated. When considered in this way (Table 6), there were conspicuous dose responses with correlation coefficients of 0.91 and 0.99.

Approaches for evaluating survival of stem spermatogonia, and using such data for predicting the eventual restoration of normal spermatogenesis, have been carefully validated and described (*10,24,35*). Both conventional morphometric analysis of surviving stem spermatogonia and enumeration of homogenization-resistant spermatid nuclei at appropriately selected intervals following exposure to an agent are valid approaches.

Evaluation of Epididymal Function

A variety of approaches could be utilized for evaluation of epididymal function (Table 2). For the immediate future, such evaluations probably will be restricted to expressing sperm from the cauda epididymidis (by mincing in a physiological salt solution) and evaluating the motility and morphology of the sperm. Composition of the buffer probably is not important for the evaluations of spermatozoal morphology, provided the osmolality does not induce artifacts such as swelling of the plasma and outer acrosomal membranes or coiling of the tail. With advances in image analysis, it is probable that commercial systems to evaluate morphology of sperm in stained smears automatically will be available soon.

For evaluation of spermatozoal motility, selection of the medium is important. Progressive spermatozoal motility may not be initiated upon dilution of epididymal spermatozoa in certain buffers, while dilution of other aliquots of the same sperm into a different buffer results in a high percentage of progressively motile sperm (*36,37*).

When evaluating spermatozoal motility, temperature control is important since both the percentage of motile sperm and spermatozoal velocity are temperature-dependent. Values based on observations at 18 to 22 °C typically are much lower than values for observations at 37°C. Consequently, if it is deemed worthwhile to evaluate spermatozoal motility, an investment should be made in equipment to control temperature adequately (slidewarmer and electrically heated microscope stage or similar device) and a phase-contrast microscope.

Instruments are available that enable automatic, computer assisted evaluation of spermatozoal motility. In addition to determining the percentage of motile sperm, some systems also allow measurement of velocity of movement, linearity of the path taken by sperm, and characteristics of head displacement or flagellar movement. It remains to be established if this information is useful for evaluating effects of drugs on sperm.

Role of Artificial Insemination

Normal males ejaculate ≥ 10-fold more sperm than are needed to achieve maximum fertility and litter size. Consequently, the number of fertile sperm ejaculated could be reduced by ≥ 90% before a reduction in fertility might be detected. This fact has been established for common animal models (*7*) and serves as the physiologic basis for the artificial insemination of millions of animals. For certain species, artificial insemination deposits

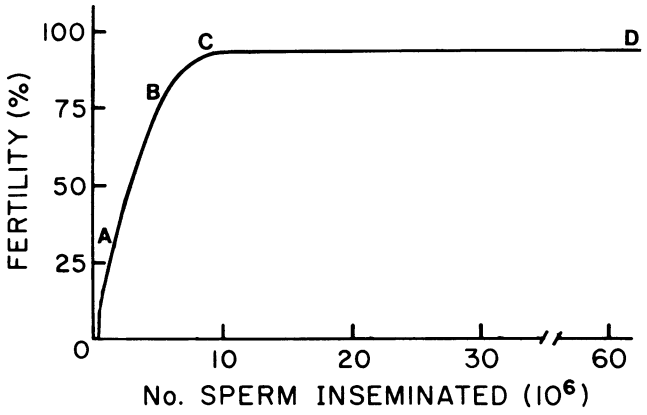

FIGURE 3. Dose-response curve for fertility as a function of number of sperm inseminated for a hypothetical strain for an animal model. Points *A* and *B* designate the sensitive area of the dose response curve; the insemination dose should be selected so that fertility with control males is near point *B*. This critical number of sperm also should be used for inseminations using sperm from treated males. Point *C* designates the number of sperm needed for maximum fertility and point *D* the number of sperm ejaculated by a normal male during natural mating.

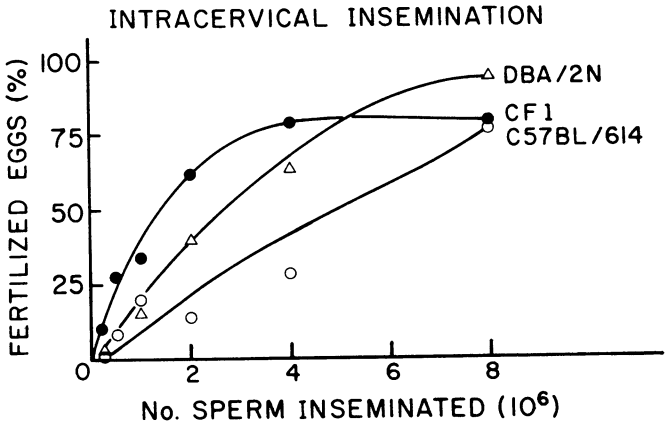

FIGURE 4. Dose-response curves for fertility as a function of number of sperm inseminated intracervically for three strains of mice. Modified from Robl and Dziuk (*38*).

sperm in the uterus rather than the cervix or vagina, which may minimize, but certainly does not eliminate, loss of sperm from the female reproductive system. Figure 3 shows fertility as a function of the total number of sperm deposited by copulation or artificial insemination. The normal male deposits a number of sperm equivalent to the extreme right end of the figure, or possibly more sperm. If a male deposits, by natural mating or artificial insemination, a number of fertile sperm greater than that equivalent to point *C* in Figure 3, normal fertility should result even if this number represented a 20, 50, or 90% reduction from the number of fertile sperm normally deposited. Therefore, with natural mating, there is a high probability that the experiment will not detect a depression of fertility except when the production and deposition of fertile sperm by copulation is drastically reduced.

If the reader mentally transforms the data depicted in Figure 3 by expanding the left portion of the x-axis, the resulting visual image will be recognized as a familiar standard curve for a biochemical assay. Obviously, when analyzing any chemical by a standard curve technique, values for unknowns must be on the portion of the standard curve where there is a reasonably linear slope. The same is true of fertility tests. Consequently, use of artificial insemination is essential to allow deposition of a "critical total number of sperm" from control animals and insemination of the same total number of sperm from treated animals. The total number of sperm to inseminate should be selected so that fertility values for control animals are near or to the left of point *B* but lie above point *A*. Under these conditions, if the agent results in a slight decrement in the percentage of fertile, normal or motile sperm, there is a high probability that a decrease in fertility will be detected if the same critical total number of sperm is inseminated for the treated and control animals.

The critical total number of sperm to be inseminated must be established for each species and strain of animals used. There are strain differences for the critical number of sperm to be inseminated into each female (Fig. 4). In addition, the inherent fertility of the female strain also affects the critical number of sperm.

Based on data such as those in Figures 3 and 4, it should be obvious that artificial insemination is a much more sensitive approach to fertility testing than use of natural mating (*39*). However, natural mating does have one distinct advantage. Males can be observed during natural mating to quantify their sexual behavior. The fact that natural mating has occurred is a demonstration that the male is able to copulate. Thus, even when the period of cohabitation of males with females is not monitored, the appearance of ejaculation plugs or the onset of pregnancy provide crude evaluations of sexual interest and copulatory ability.

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